Remarks

Consideration of this Application is respectfully requested.

Upon entry of the foregoing amendment, claims 2-5 and 9-10 are pending in the application, with claims 2 and 9 being the independent claims. Claim 2 has been amended in order to more clearly and precisely define the subject matter which Applicants regard as the invention. Support for the amendment can be found throughout the specification and the original claims. Specifically, support can be found in the specification, *inter alia*, at page 19, lines 21-25. Claims 6-8 and 12 have been canceled. These claims were in groups that were not elected after issuance of the Restriction Requirement. Applicants retain the right to prosecute the subject matter of claims 6-8 and 12 in continuing applications. The amendments do not add new matter. Applicants respectfully request that the amendments be entered.

Based on the above amendment and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

Status of the Application

On October 26, 2000, in the parent application, a final Office Action, rejecting claims 2-3 under 35 U.S.C. § 102(a) and claims 4-5 and 9-10 under 35 U.S.C. § 103 was issued. (Paper No. 15.) Applicants responded to the final Office Action on March 26, 2001. On April 10, 2001, an Advisory Action was issued maintaining the rejection of claims 2-3 under 35 U.S.C. § 102(a) and the rejection of claims 4-5 under 35 U.S.C. § 103. (Paper No. 20.) Applicants have filed herein a continuing prosecution application (CPA) and a preliminary amendment, which responds to the Examiner's rejections of Paper No. 20.

The Telephonic Interview of July 3, 2001

Applicants thank the Examiner for the telephonic interview held with the undersigned on July 3, 2001.

Claim Rejections Under 35 U.S.C. § 102

The rejection of claims 2 and 3 under 35 U.S.C. § 102 as allegedly being anticipated by Vito *et al.*, *J. Biol. Chem.* 271(49):31025-31028 (1996) has been maintained. (Paper No. 20, page 2.)

Claim 2 has been amended to further clarify the claimed invention. Specifically, claim 2 has been amended so that it is clear that the claimed antibody specifically binds a purified 20 kDa presenilin-2 C-terminal fragment *but does not bind presenilin-2*. Support for the amendment can be found, *inter alia*, at page 19, lines 20-25 of the specification. Generating antibodies specific for a proteolytically cleaved fragment of a full-length protein, and that do not bind the full-length protein is within the skill of the art as evidenced, for example, by Hughes *et al. J. Biol. Chem.* 267(23): 16011-14 (1992) (Exhibit A) who teach antibodies specific to a particular fragment of cleaved link protein (LP) but not uncleaved LP and Lark *et al.*, 40th Ann. Meeting, Orthopaedic Res. Soc., Feb. 21-24 (1994) (Exhibit B), who teach antibodies that bind a particular fragment of the cleaved aggrecan protein but do not bind the full-length aggrecan protein.

"For a prior art reference to anticipate in terms of 35 U.S.C. § 102, every element of the claimed invention must be identically shown in a single reference." *In re Bond*, 15 USPQ2d 1566, 1567 (Fed. Cir. 1990). Vito *et al.* do not teach every element of the claimed invention and, therefore, cannot anticipate the claims. Specifically, the antibodies of Vito *et al.* are capable of binding to presenilin-2 whereas the claims require that the antibody does not bind to presenilin-2. As discussed in Applicants' Amendment and Reply Under 37 C.F.R. § 1.111, the polyclonals of Vito *et al.* can bind to any presenilin-2 having amino acids 341-377 and 438-448, i.e., full-length presenilin-2, normal C-terminal fragment of presenilin-2 (25 kDa), and the presenilin-2 C-terminal fragment of the captioned application (20kDa), as well as ALG-3 (by the polyclonal specific for amino acids 438-448

of presenilin-2). Thus, Vito *et al.* do not teach the claimed invention. Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw the rejection.

The rejections of claim 4 under 35 U.S.C. § 103 as allegedly unpatenable over Vito *et al.* in view of Dalbow *et al.*, U.S. Patent No. 4,116,776 and claim 5 under 35 U.S.C. § 103 as allegedly unpatentable over Vito *et al.* in view of Janeway *et al.*, Immunobiology, New York, Current Biology, 1997 have also been maintained.

In order to establish a prima facie case of obviousness under 35 U.S.C. § 103, the references cited must teach all of the claim limitations. MPEP § 706.02(j). The defects of Vito *et al.* have been discussed above. Neither Dalbow *et al.* nor Janeway *et al.* cure the defects of Vito *et al.* by teaching an antibody that specifically binds a purified 20 kDa presenilin-2 C-terminal fragment but does not bind presenilin-2. Accordingly, a prima facie case of obviousness has not been made. Thus, Applicants respectfully request that the Examiner reconsider and withdraw the rejection of claims 4 and 5 under 35 U.S.C. § 103.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that they have addressed and traversed the rejections maintained in the Advisory Action issued on April 10, 2001 (Paper No. 20) and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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Version with markings to show changes made

In the claims:

2. (Amended) An antibody having binding affinity that is specific [only] to a purified 20 kDa presenilin-2 C-terminal fragment (PS2-CTF), wherein said antibody does not bind presenilin-2.

Claims 6-8 and 12 have been canceled.

Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Monoclonal Antibodies Recognizing Protease-generated Neoepitopes from Cartilage Proteoglycan Degradation

APPLICATION TO STUDIES OF HUMAN LINK PROTEIN CLEAVAGE BY STROMELYSIN*

(Received for publication, May 29, 1992)

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Monoclonal antibodies were raised that specifically recognize the NH_2-terminal neoepitope sequence present in link protein cleavage products derived from stromelysin-degraded proteoglycan aggregate. Competitive enzyme-linked immunosorbent assay, using synthetic peptides as inhibitors, showed that one of these antibodies (CH-3) required, for antibody recognition, the free NH_2-terminal amino acid isoleucine (residue 17 of the intact protein) in the sequence NH_2-IQAENG at the stromelysin cleavage site of link protein 3. Human proteoglycan aggregate was digested with recombinant human stromelysin, bovine chymotrypsin, bovine trypsin, and porcine elastase, and their respective link protein degradation products were tested for immunoreactivity with antibody CH-3. Only stromelysin- and chymotrypsin-generated link protein 3 were recognized by antibody CH-3. Both of these enzymes generate link protein NH_2 termini with the sequence ^{17}IQAENG. . .; hence these studies indicated that monoclonal antibody CH-3 recognized this neoepitope sequence in only specific proteolytically modified link protein molecules. Since the occurrence of link protein 3 increases with aging, the incidence of CH-3 epitope in proteoglycans isolated from human knee articular cartilage of individuals of different ages was investigated. The prevalence of CH-3 epitope was found to be highest in newborn and adolescent articular cartilage samples. However, little CH-3 epitope was detected in older adult cartilage, although considerably more link protein 3 was present in these samples. These results suggest that additional proteolytic agents are responsible for the increased occurrence of link protein degradation products with aging.

Mechanisms of proteoglycan breakdown in connective tissues are complex and involve multiple agents and pathways (1). This is indicated by several studies examining the turnover of matrix macromolecules in both articular cartilage and intervertebral disc (2–6). Recently, amino acid sequence analysis of cartilage proteoglycan breakdown products (4–6) has contributed to the identification of putative sites of catabolism within these macromolecules. The occurrence of the different sequences at these cleavage sites indicates that several different agents must be involved in proteoglycan turnover. At present the identity and the source of the agents responsible for this degradation and the extent to which each agent is contributing to the turnover of these molecules have not been definitively established. However, since many of the proteolytic cleavage sites in proteoglycan are now known, we hypothesized that it should be possible to use immunological procedures to both identify and quantitate the occurrence of an agent-specific cleavage site in these molecules. It has been demonstrated previously in the case of fibrinogen (7) that on cleavage of a protein, the new NH_2 and COOH termini produced have different properties, in terms of antibody recognition, than the same sequences present in the intact protein. These findings suggest that it should be possible, therefore, to produce antibodies specific for the products of proteoglycan aggregate components that have been cleaved by specific proteinases. Proteinases can cleave all susceptible peptide bonds without bias for the protein in which they occur. Thus, in principle, any matrix protein may be used as a marker for proteinase action. However, in practice, it is necessary that the protein be cleaved by physiologically relevant proteinases, and at least one recognizable degradation product must remain localized within the tissue. Link protein from cartilage proteoglycan aggregate satisfies these criteria (8).

Link protein (LP)[1] is a small globular protein which stabilizes the interaction between the cartilage proteoglycan monomer (aggrecan) and hyaluronan (9, 10). This interaction results in the formation of the large proteoglycan aggregates that endow cartilage with its property of resistance to compression under load (11, 12). Link protein isolated from human articular cartilage can be separated into three components with estimated molecular masses of 48, 44, and 41 kDa, which are referred to as LP1, LP2, and LP3, respectively (13–15). Peptide mapping and amino acid sequence analysis indicated that the different forms of link protein are derived from the same protein core (16–19). The difference in electrophoretic migration of LP1 and LP2 has been shown to be due to the differential substitution of *N*-linked oligosaccharides on the two different link protein components (15–17, 19, 20). Nguyen *et al.* (18) have demonstrated that human LP1 and LP2 differ only by the presence of an *N*-linked oligosaccharide at residue 6 in LP1. LP3 is a proteolytic cleavage product of either LP1 or LP2, which appears to have similar functional properties to those of the intact molecule (8, 15). The NH_2-terminal region of LP1 and LP2, between residues 10 and 30, appears to be extremely susceptible to proteolytic attack, and

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[1] The abbreviations used are: LP, link protein; βA, β-alanine; ELISA, enzyme-linked immunosorbent assay; EIA, enzyme immunoassay; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

the cleavage positions for many proteinases have been determined (8). When LP3 preparations isolated from normal and diseased human articular cartilage were analyzed, three distinct NH_2 termini were found. By comparison with the *in vitro* cleavage data, the proteinases of physiological relevance thought to be responsible for generating these new NH_2 termini were postulated to be stromelysin, cathepsin G, or cathepsin B (8). Human cartilage LP3 is therefore present as a family of molecules, the identity of which depends on the proteinase responsible for its generation. Since the generation of each LP3 produces a unique NH_2 terminus, we propose the nomenclature LP3(n) for each defined LP3, where n represents the sequence position of the NH_2 terminus of the molecule relative to that in the native protein. Thus, LP3 generated by the action of stromelysin is denoted as LP3(17).

In this paper we describe a novel approach, using monoclonal antibody technology, to identify proteolytic agents responsible for the degradation of matrix macromolecules, in normal turnover and in disease, by preparing antibodies specific for the cleavage sites produced by different proteinases in matrix molecules. As an example, this study was directed toward examining the role that stromelysin plays in the degradation of link protein during cartilage proteoglycan catabolism.

EXPERIMENTAL PROCEDURES

Materials—Pristane (2,6,10,14-tetramethylpentadecane) was obtained from Aldrich and 1,10-phenanthroline from Mallinckrodt Chemical Works. Titertek EIA plates were purchased from Flow Laboratories. Alkaline phosphatase-conjugated rabbit anti-mouse Fab_2 used in ELISA was obtained from Southern Biotechnology Inc., Birmingham, AL. Alkaline phosphatase-conjugated second antibody and substrate used in the Western blot analysis were obtained from Promega as the Protoblot Western blot AP system (catalog no. W3920). Nitrocellulose (0.2-μm pore size) was obtained from Bio-Rad and polyvinylidene difluoride membrane (Immobilon) from Millipore. Human recombinant prostromelysin was a generous gift from Dr. Gillian Murphy, Strangeways Research Laboratories, Cambridge, U.K. Bovine chymotrypsin (tosyllysine chloromethyl ketone-treated), bovine trypsin (tosylphenylalanine chloromethyl ketone-treated), and porcine pancreatic elastase were all purchased from Sigma. Monoclonal antibody 8-A-4 was prepared as ascitic fluid and also purchased from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). This antibody recognizes a linear amino acid sequence epitope in the paired tandem repeat domains of link proteins isolated from many animal species (16). All other chemical reagents used that are not specifically mentioned above were of analytical grade and obtained from either Sigma or Fisher Scientific.

Preparation of Antigens for Immunization and Screening—Peptides were synthesized, at a 0.5-mmol scale using standard Fmoc (9-fluorenylmethoxycarbonyl) chemistry, on an Applied Biosystems model 431A solid phase peptide synthesizer. Crude peptides were purified by reverse phase chromatography (Prep-10 Aquapore C8 column, Applied Biosystems) using an acetonitrile gradient in 0.1% trifluoroacetic acid. Peptides synthesized were IQAENGGC, IQAENG(βA)C, Ac-IQAENG(βA)C, IHIQAENGGC, HIQAENGGC, QAENG(βA)C, AENGPHGGC, and DHLSDNYTGGC. Most peptides contained a COOH-terminal spacer amino acid, glycine, followed by a cysteine residue that was used as the coupling site for preparation of peptide-protein conjugates. In some peptides used to probe antibody specificity, β-alanine (βA) was used as a substitute for the glycine spacer residue.

The bifunctional reagent, *N*-hydroxysuccinimidylbromoacetate, was synthesized as described by Bernatowicz and Matsueda (21). For coupling, 0.2 ml of an 65 mg/ml solution of this bifunctional reagent in dimethylformamide was added, dropwise, with continuous stirring to 2.25 ml of ovalbumin (22 mg/ml) dissolved in 0.1 M potassium phosphate, pH 7.5, containing 1 mM EDTA at 4 °C. The mixture was then allowed to equilibrate to room temperature over a 30-min time period. The activated ovalbumin was separated from unreacted reagents by gel filtration using a Sephadex G-25 column (25 × 1.5 cm) eluted with the EDTA-phosphate buffer described above. The synthetic peptide to be coupled to the carrier protein was dissolved in water (0.4 ml) at a concentration of 6 mM and added to 0.4 ml of the Sephadex G-25 eluate peak fraction containing the activated ovalbumin. The mixture was initially shaken gently under nitrogen for 2 h at room temperature, followed by continued incubation overnight at 4 °C. Unreacted bromoacetate groups were blocked by the addition of mercaptoethanol (1 μl), and the solution was dialyzed exhaustively against PBS. The success of the coupling reaction was determined by observation of a decrease in electrophoretic mobility of the peptide-ovalbumin conjugate, on SDS-PAGE, relative to a cysteine-ovalbumin conjugate prepared in the same manner. The identity of the peptide on the conjugate was confirmed by peptide sequencing, following SDS-PAGE and electroblotting onto a polyvinylidene difluoride membrane (22).

Immunization and Fusion—The antigen used for immunization was a peptide-ovalbumin conjugate, consisting of the synthetic peptide IQAENGGC coupled to the carrier protein. A single 4–6-week-old female BALB/c mouse was immunized with the ovalbumin-peptide conjugate. Procedures for immunization, cell fusion, and hybridoma selection were as described by Caterson *et al.* (23, 24). After 10–14 days in culture, the wells were visually inspected for the presence of viable hybridomas. Aliquots from each of the hybridoma-containing wells were tested in ELISA for the presence of mouse immunoglobulin with specificity directed against the original immunizing antigen. These hybridoma supernatants were also tested for antibody activity against ovalbumin-peptide conjugates containing an unrelated peptide sequence, in order to distinguish hybridomas that produced antibodies recognizing the synthetic peptide from those recognizing epitopes in either the coupling region of the conjugate or the carrier protein. Hybridomas from wells producing antibody directed against epitopes in the synthetic peptide component of the ovalbumin-peptide conjugate were then expanded and subcloned by limiting dilution. Media from the resultant monoclonal hybridomas were retested in ELISA for their production of immunoglobulin directed against the original antigen, then expanded in cell culture, and used for ascites production. Ascites were produced by the intraperitoneal injection of 10^6–10^7 cells into female retired breeder BALB/c mice that had been previously primed with an intraperitoneal injection of 0.5 ml of Pristane 1–2 days earlier. Ascitic fluid was usually harvested 2–3 weeks after the hybridoma injection and stored in the presence of 0.02% NaN_3. The antibody isotype was determined using an Isotype screening kit supplied by Southern Biotechnology Inc., Birmingham, AL.

ELISA Screening Procedures—Antigens were dissolved in PBS-azide (0.14 M NaCl, 0.01 M sodium phosphate, pH 7.2, containing 0.02% NaN_3) and coated at a concentration of 3 μg/ml on 96-well Titertek EIA plates by passive absorption either overnight at 4 °C or for 3 h at 37 °C. The plates were washed with PBS-azide, and the unreacted sites were blocked by the addition of EIA buffer (1% BSA in PBS-azide; 200 μl/well) followed by incubation for 1 h at 37 °C. The plates were then washed three times with PBS-azide, and 200 μl of each hybridoma culture supernatant was added to appropriate wells of the EIA plates and incubated for 1 h at 37 °C. The plates were washed four times with PBS-azide, followed by the addition of 200 μl of a 1:500 dilution of enzyme-linked second antibody solution (alkaline phosphatase-conjugated rabbit anti-mouse Fab_2) and incubation for 1 h at 37 °C. The plates were washed five times with Tris-saline buffer (0.2 M NaCl, 0.05 M Tris-HCl, pH 7.4) and then incubated at 37 °C with alkaline phosphatase substrate (*p*-nitrophenyl phosphate, 1 mg/ml in 0.126 mM $MgCl_2$, 0.86 M diethanolamine, pH 9.8) until optimal color development occurred (usually 30–90 min). The color was quantified by measuring the absorption at 405 nm on a Titertek Multiskan (Flow Laboratories).

ELISA Inhibition Assays—The immunizing peptide antigen was dissolved in PBS-azide and coated onto EIA plates at 3 μg/ml (200 μl/well), then blocked in EIA buffer, as described above. A panel of peptides, all at 1 mg/ml, was serially diluted in EIA buffer (concentration range from 0 up to 30 μg/100 μl) and incubated at room temperature for 1 h with monoclonal antibody CH-3 (final dilution of 1:10,000 in 200 μl) prior to incubation for 1 h at 37 °C in the appropriate well of the antigen-coated and blocked EIA plate. The plates were washed and incubated with secondary antibody as described above in the direct ELISA. Substrate was then added, and the plates were incubated at 37 °C until the well that contained no competing antigen (0 μg/ml) gave an absorbance reading of approximately 1.0 at 405 nm. This reading was taken as 100%.

Proteinase Digestion of Cartilage Proteoglycan Aggregate—Proteoglycan aggregate isolated from normal human knee articular cartilage was prepared by associative CsCl equilibrium density gradient cen-

trifugation (13). The proteoglycan aggregate fraction (A1)[2] was subjected to proteolytic digestion with either chymotrypsin, trypsin, or pancreatic elastase. Digestion mixtures (0.1 ml) contained 2 mg/ml proteoglycan aggregate in 0.1 M Tris-HCl, pH 7.5, and 20 μg/ml of either of the three proteinases listed above. Following incubation at 37 °C for 4 h, the enzymes were inactivated by addition of an equal volume of SDS-PAGE sample buffer (without mercaptoethanol) and heated immediately in a boiling water bath for 3 min. The proteoglycan aggregate was also subjected to digestion with recombinant prostromelysin, which had been preactivated by *p*-aminophenyl mercuric acetate treatment (25). Incubations contained 1 mg/ml proteoglycan aggregate in 0.1 M Tris-HCl, pH 7.5, containing 10 mM $CaCl_2$ and 10 μg/ml stromelysin and were allowed to proceed at 37 °C for 24 h and then terminated as above. Link proteins were separated by SDS-PAGE, electroblotted onto nitrocellulose, and immunolocalized with monoclonal antibodies using procedures described below.

Isolation and Analysis of Cartilage Matrix Proteins—Macroscopically normal human articular cartilage was obtained from the distal femur at autopsy, within 16 h of death, from donors aged 6 weeks, 2 years, 10 years, 23 years, and 58 years, respectively. The tissue was finely diced and extracted with 4 M guanidinium chloride-containing proteinase inhibitors (26), and the protein-rich components of the cartilage extracts were isolated by direct dissociative CsCl equilibrium density gradient centrifugation using a starting density of 1.50 g/ml (14). After centrifugation, the tubes were divided into three fractions (D1-D3),[2] which were then dialyzed and freeze-dried. Cartilage matrix proteins in the low buoyant density D3 fraction were dissolved at 2 mg/ml in 4 M guanidinium chloride, 0.1 M sodium acetate, pH 6.3, and then dialyzed at room temperature against two changes of 0.125 M Tris-HCl, pH 6.8, containing 0.1% SDS. Samples (50 μg/well) were subjected to SDS-PAGE, followed by electroblotting onto nitrocellulose membranes. Link proteins were immunolocalized with monoclonal antibodies 8-A-4 and CH-3 using procedures described below.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—Samples containing native and degraded link protein were electrophoresed on 10% polyacrylamide slab gels in SDS using procedures described by Laemmli (27). After electrophoresis, the fractionated proteins were electrophoretically transferred to nitrocellulose membranes. The transfer sheet was incubated in blocking solution (3% (w/v) bovine serum albumin in PBS-azide) overnight, then incubated with a 1:2000 dilution of monoclonal antibody 8A4 or a 1:100 dilution of CH3 in TBST (0.05% Tween 20 in 0.15 M NaCl, 0.01 M Tris-HCl, pH 8.0) for 30 min at 37 °C. After 3 washes with TBST buffer, the nitrocellulose sheets were incubated for 30 min at 37 °C with a 1:7500 dilution of an alkaline phosphatase-conjugated rabbit anti-mouse second antibody in TBST buffer. The nitrocellulose was then thoroughly washed in TBST buffer and then washed in one change of alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5) before addition of freshly prepared alkaline phosphatase substrate solution (66 μl of nitro blue tetrazolium (50 mg/ml in 70% dimethylformamide) and 33 μl of 5-bromo-4-chloro-3-indoyl phosphate (50 mg/ml in dimethylformamide) in 10 ml of alkaline phosphatase buffer). In general, the immunoblots were incubated for 10-30 min at room temperature to achieve optimum color development.

RESULTS

Characterization of a Monoclonal Antibody Specific for LP3(17)—Mice were immunized with a synthetic peptide conjugate containing the first 6 residues of the NH_2-terminal neoepitope produced from cleavage of native link protein by stromelysin. Preliminary screening of hybridoma supernatant specificity in direct ELISA identified six clones (denoted CH-3 to CH-8, respectively) that recognized the immunizing antigen. These antibodies showed no reactivity with unrelated synthetic peptide conjugates nor with the carrier protein. Hybridoma clone CH-3 showed the strongest reactivity in the preliminary screening and thus was processed further. The CH-3 hybridoma cell line was cloned by limiting dilution, and its antibody isotype determined as being an IgG_1 heavy chain and κ light chain.

The reactivity of monoclonal antibody CH-3 against a series of unconjugated peptides, related to the immunizing antigen but having modifications at both the NH_2- and COOH-terminal amino acids, was tested in competitive ELISA (Fig. 1). These analyses showed that the unconjugated immunizing peptide and the same peptide with a modified COOH-terminal amino acid sequence both gave 50% of the maximum absorbance at a concentration of 25 μg/ml peptide (Fig. 1, *open symbols*). Acetylation of the peptide resulted in a marked reduction in inhibition, while removal of the NH_2-terminal amino acid (isoleucine) resulted in a complete loss of this inhibition. Similarly, NH_2-terminal addition of further residues from the link protein sequence (Fig. 2*A*) gave peptides that were unable to compete with the immunizing sequence, even when an alternative NH_2-terminal isoleucine residue

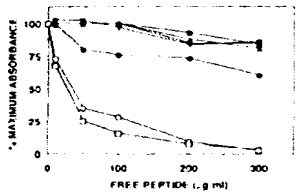

FIG. 1. **Competitive inhibition ELISA assay using monoclonal antibody CH-3.** EIA microtiter plates were coated with the ovalbumin-synthetic peptide (IQAENGGC) conjugate used as the immunizing antigen. Synthetic peptides used as competing antigens in the inhibition ELISA were: ○, IQAENGGC; □, IQAENG(βA)C; ●, Ac-IQAENG(βA)C; ■, IHIQAENGGC; ▲, HIQAENGGC; ▼, QAENG(βA)C; and ◆, AENGPHGGC.

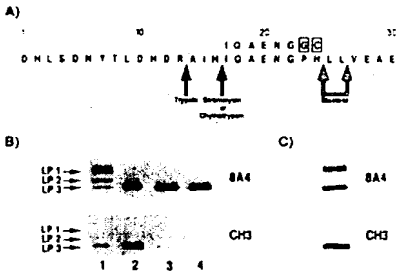

FIG. 2. **Characterization of monoclonal antibody CH-3.** *A*, diagrammatic representation of the NH_2-terminal amino acid sequence (residues 1-30) of human link protein, illustrating (*below*) the cleavage sites of trypsin, chymotrypsin, stromelysin, and elastase, and (*above*) the peptide used as the immunogen. *Boxed residues* indicate the linker region of the synthetic peptide antigen. *B*, samples of human proteoglycan aggregate digested with proteinases (10 μg of proteinase/mg of proteoglycan aggregate, 4 h) were analyzed by SDS-PAGE and Western blotting. Link protein components were visualized using monoclonal antibodies 8-A-4 or CH-3 as indicated. *Lane 1*, control; *lane 2*, chymotrypsin; *lane 3*, elastase; *lane 4*, trypsin. *C*, human proteoglycan aggregate digested with *p*-aminophenyl mercuric acetate-activated recombinant prostromelysin (10 μg of proteinase/mg of proteoglycan aggregate, 24 h) and then analyzed as in *B*.

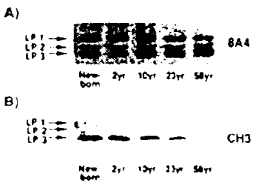

FIG. 3. **Changes with age in the CH-3 epitope in human articular cartilage.** Cartilage proteins from the D3 fraction of dissociative CsCl density gradients were subjected to SDS-PAGE and Western blotting. Link protein components were visualized using monoclonal antibody 8-A-4 (*A*) and monoclonal antibody CH-3 (*B*).

[2] A1 and D1-D3 as used in Ref. 12.

was present (Fig. 1, *closed symbols*). These results demonstrated the importance of the free NH_2-terminal isoleucine residue and the dependence on the specific amino acid sequence adjacent to this residue in the epitope recognized by CH-3.

The specificity of monoclonal antibody CH-3 for human LP3(17) was demonstrated by immunolocalization studies on specific proteinase digestion products of human articular cartilage proteoglycan aggregate (Fig. 2). Following SDS-PAGE and electroblotting, immunolocalization with monoclonal antibody 8-A-4 (which recognizes internal epitopes in link protein) visualized LP1, LP2, and LP3 in the undigested preparation, with LP1 and LP2 being most abundant and LP3 being present as a minor component. However, digestion with chymotrypsin, elastase, or trypsin resulted in complete conversion of LP1 and LP2 to LP3 products, the sequences of which had been determined previously (Fig. 2*A*). In contrast to 8-A-4, monoclonal antibody CH-3 gave a weak LP3 band in the undigested sample and did not react with the trypsin or elastase digestion products. However, chymotrypsin digestion, which is known to produce LP3(17), gave a strong reaction (Fig. 2*B*). Similarly, digestion with recombinant stromelysin produced a strong CH-3 reactive band (Fig. 2*C*). NH_2-terminal sequencing of this recombinant stromelysin-generated LP3 gave the expected sequence. These data demonstrate the specificity of antibody CH-3 for the sequence NH_2-IQAENG... in the native LP3 preparations.

Changes with Age in LP3(17) Abundance—In order to evaluate the importance of stromelysin action in cartilage changes associated with aging, the occurrence of CH-3 neoepitope in link proteins from human articular cartilage extracts from various individuals of different ages was examined (Fig. 3). Immunolocalization analysis, using antibody 8-A-4, indicated that the relative abundance of LP3 was found to increase with age at the expense of the LP1 and LP2 components, in agreement with previous results (14). In contrast, however, the proportion of LP3(17), as visualized using the monoclonal antibody CH-3, was found to decrease with the age of the individual.

DISCUSSION

Results from several groups indicate that the mechanism of proteoglycan catabolism in connective tissues is complex. Protein sequencing studies have documented many of the cleavage sites that result from catabolism of the proteoglycan aggregate components, aggrecan (4–6) and link protein (8, 18). In recent years, reports in the literature have focused on the metalloproteinases stromelysin and collagenase as being the major agents involved in cartilage proteoglycan degradation (28). However, it is clear that many of the proteolytic agents responsible for this catabolism still remain to be identified (4–6, 8). Research in our laboratories has been directed toward using monoclonal antibody technology to identify specific *in vivo* cartilage proteoglycan breakdown products as a means of discriminating between the large number of proteolytic agents that are potentially involved in this process.

The present work demonstrates the feasibility of this approach. Monoclonal antibodies specific for the stromelysin cleavage site in the degraded link protein present in catabolized cartilage proteoglycan aggregate have been produced. One of these antibodies (CH-3) recognized only the neoepitope sequence containing an NH_2-terminal isoleucine (residue 17 of the intact protein) and its adjacent amino acids but not the identical sequence present in the undegraded link-protein. This finding suggests that monoclonal antibody CH-3 can be used to distinguish between the degradative action of stromelysin *versus* other proteinases in matrix turnover, since of the physiologically relevant proteinases studied so far only stromelysin cleaves at this position. Our studies using monoclonal antibody CH-3 also provided evidence suggesting that the major proportion of LP3 in the adult is not a final degradation product of stromelysin, even though during aging an increasing amount of the link protein in human articular cartilage is present in the LP3 form. This indicates that catabolic agents other than this metalloproteinase contribute to link protein degradation and presumably to the turnover of matrix molecules in general in normal adult cartilage. It still remains to be established whether or not multiple proteolytic agents (including stromelysin) are involved in this process.

These results indicate that neoepitope monoclonal antibody technology is a powerful tool for identifying the unique degradation products that result from the action of individual proteolytic agents in extracellular matrix degradation. In addition to their application in immunoblotting, these reagents can also be used for immunohistochemical localization and quantitative immunoassay. Thus, this methodology has great potential for discriminating between the different mechanisms of cartilage metabolism operating in health and disease.

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DEVELOPMENT OF AN ANTIBODY AGAINST A METALLOPROTEINASE GENERATED NEO-EPITOPE IN HUMAN AGGRECAN

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INTRODUCTION: Stromelysin-1 (SLN), is elevated in synovial fluids of patients with osteoarthritis and rheumatoid arthritis[1]. One approach to determine if SLN has activity in cartilage, is to develop antibodies to localize and quantify matrix degradation products of the enzyme. SLN cleaves human aggrecan between Asn^{341} and Phe^{342} in the interglobular domain (IGD) region of the molecule[2]. An anti-peptide antibody was generated against the C-terminal sequence of the hyaluronan binding region (HABR) generated after SLN cleavage and a quantitative radioimmunoassay (RIA) developed. The specificity, sensitivity and utility of this antibody are described.

MATERIALS AND METHODS: The peptide $CNleFVDIPEN^{341}$, corresponding to the C-terminus of the SLN-generated HABR, was conjugated to bovine thyroglobulin and an antiserum generated in rabbits. The serum was used for development of a quantitative RIA and for Western blotting of aggrecan and aggrecan fragments. ProSLN, procollagenase (proCLN), and progelatinase (proGLN) A were recombinant human enzymes supplied by Celltech, Ltd. Human GLN B was supplied by G. Goldberg (Wash. Univ.). ProSLN was activated using trypsin or APMA[3]. ProCLN and both proGLNs were activated using APMA. Cathepsins (Cat) B and G were purchased from Athens Research. Human leukocyte elastase (HLE) was supplied by B. Green (MRL).

RESULTS: The RIA using the rabbit polyclonal anti-FVDIPEN antiserum and has a limit of detection of ~35 pM (Fig 1). The sequence, VDIPEN, is required for full recognition by the antibody. When the C-terminal Asn is truncated from the sequence or when the Phe, in position 342 is present, there is 1,000 fold loss in recognition by the antibody. These data indicate that the antiserum requires the free carboxyl on the C-terminal Asn for optimal recognition. The anti-FVDIPEN antibody does not recognize intact human aggrecan but does recognize a fragment generated after SLN cleavage both by Western blotting and RIA. The RIA recognizes cleaved aggrecan at a concentration similar to the FVDIPEN peptide. The SLN-generated HABR, terminating in $FVDIPEN^{341}$, migrates with a Mr = 50 kD on reducing SDS-polyacrylamide gels and retains its ability to bind to HA. A series of metalloproteianses including GLN and CLN have been reported to also cleave aggrecan in this site. When CLN, GLN A, GLN B, Cat B, Cat G and HLE were evaluated for their ability to generate the epitope, the only enzyme other than SLN which generated the epitope was GLN A (Fig 2). The anti-VDIPEN antiserum recognizes SLN-generated HABR from rabbit, human, guinea pig, and mouse aggrecan.

DISCUSSION: An anti-peptide antibody has been raised against the C-terminal sequence of the HABR generated by SLN cleavage of aggrecan. The antibody requires the sequence VDIPEN for full recognition. The antiserum can be used for both RIA and Western blotting and does not detect intact aggrecan, but does detect SLN-cleaved aggrecan from a number of species. It appears that the only other matrix metalloproteinase capable of generating this cleavage *in vitro*, is GLN A. Because this antibody detects aggrecan fragments after enzyme cleavage in multiple species, it should be useful to detect and quantify HABR fragments generated by these enzymes as a readout of enzyme activity in man as well as animal models.

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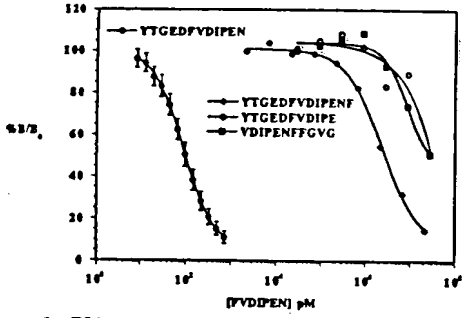

Figure 1. RIA titrations of specificity peptides.

Figure 2. Western blot of aggrecan digested with SLN (lanes1 and 3), CLN (lane 4), GLN A (lane 5) GLN B (lane 6) human leukocyte elastase (lane 7), cathepsin G (lane 8) cathepsin B (lane 9) and intact aggrecan (lanes 2 and 10), probed with anti-VDIPEN antiserum. Samples were electrophoresed on a 4-20% reducing grdient SDS-PAGE, figures on left indicate size of MW markers in kD.

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